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Isolation and characterisation of a xylanase inhibitor *Xip*-II gene from durum wheat

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ABSTRACT

Cereals contain xylanase inhibitor proteins (XIPs) which inhibit microbial xylanases from glycoside hydrolase families 10 and 11. Here, we report for the first time the isolation and characterisation of a genomic clone containing a xylanase inhibitor gene. This gene, *Xip*-II, isolated from a durum wheat genomic library (*Triticum durum* Desf.) encodes a mature protein of 307 amino acid (aa) residues that shares highest aa sequence identity (64%) with the rice RIXI xylanase inhibitor. XIP-II showed inhibition against family 11 xylanases and no chitinase activity. *In silico* analysis of the 5′ promoter region of *Xip*-II revealed sequences with similarity to known cis regulatory elements upstream from the initiation codon. In particular, the identification of a number of cis-acting elements controlling the expression of defence and seed-specific genes supports the role for this class of inhibitors in plant defence against pathogens but also provides new clues on a potential role in plant development.

1. Introduction

Endo-β-1,4-xylanases (xylanases; EC 3.2.1.8) are key enzymes in the degradation of arabinoxylans (AXs), the main non-starch polysaccharides from grain cell walls. The majority of xylanases cluster into families 10 and 11 of the glycoside hydrolase (GH) classification (CAZY database www.cazy.org) but xylanases have also been classified in GH families 5, 7, 8 and 43 (for a review, see Collins et al., 2005). Xylanases are routinely used in bread making (Butt et al., 2008), wheat gluten–starch separation (Frederix et al., 2003) and as supplements in animal feed production (Choct et al., 2004). In these applications, the observed effects are largely dependent on the variability in xylanase specificity towards AXs, which can be attributed to different factors such as their GH family origin, sensitivity to protein inhibitors, modular architecture, and substrate selectivity (Berrin and Juge, 2008).

To date, two main types of proteinaceous xylanase inhibitors have been identified in monocots, namely the TAXI [*Triticum aestivum* xylanase inhibitor]-type and XIP [xylanase inhibiting protein]-type xylanase inhibitors (Juge and Delcour, 2006). These inhibitors are widely represented in cereals (rye, barley, maize, rice, durum and bread wheat) where they occur as multi-isoforms (Goesaert et al., 2004). XIP-type inhibitors are glycosylated monomeric proteins with a molecular mass of 29 kDa and p*I* values of 8.7–8.9 whereas TAXI-type xylanase inhibitors are high p*I*, non-glycosylated proteins occurring in two molecular forms, a monomer of approximately 40 kDa, and a heterodimer composed of two disulfide-linked subunits of 29 and 11 kDa (Juge and Delcour, 2006). The best characterized inhibitors in terms of structure and function are the XIP-I and TAXI-I proteins from wheat *T. aestivum*. Recently, a third type of xylanase inhibitor, i.e. thaumatin-like xylanase inhibitor (TL-XI), has been identified in wheat with a molecular mass of approximately 18 kDa and a basic p*I* value (>9.3) (Fierens et al., 2007). These proteins have been detected and characterized by their abilities to inhibit microbial xylanases in vitro. XIP-type inhibits microbial GH10 and GH11 xylanases whereas TAXI-type and TL-XI inhibitors exclusively inhibit bacterial and fungal GH11 xylanases (Juge, 2006). The structural basis of their inhibition has been elucidated by X-ray crystallographic

Abbreviations: GH, glycoside hydrolase; PR, pathogenesis-related; TAXI, *Triticum aestivum* xylanase inhibitor; TL-XI, thaumatin-like xylanase inhibitor; XIP, xylanase inhibitor protein; XYNC, xylanase C from *Penicillium funiculosum*.

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analysis of XIP-I and TAXI-I in complexes with fungal xylanases (Payan et al., 2004; Sansen et al., 2004).

Xylanase action is indispensable in the infection of plants, as demonstrated using the pathogen *Botrytis cinerea* (Brito et al., 2006); TAXI and/or XIP-inhibit GH11 xylanases of the phytopathogens *Fusarium graminearum* and *B. cinerea* (Beliën et al., 2005; Brutus et al., 2005), and both TAXI and XIP genes are induced by pathogens and wounding in wheat (Igawa et al., 2004, 2005; Takahashi-Ando et al., 2007), maize (Chivasa et al., 2005) and rice (Tokunaga and Esaka, 2007), suggesting that cereal xylanase inhibitors play a role in the plant defence mechanism against pathogens (Juge, 2006).

From the phylogenetic analyses (Durand et al., 2005; Takahashi-Ando et al., 2007; Tokunaga and Esaka, 2007), it is apparent that XIP-type inhibitors evolved from the glycoside hydrolase family 18 (GH18) after the emergence of the various subfamilies of chitinases from their common ancestor (Durand et al., 2005). Despite the increasing number of cDNA sequences encoding XIP-type inhibitors from wheat (Elliott et al., 2002; Igawa et al., 2005; Takahashi-Ando et al., 2007) and rice (Durand et al., 2005; Goesaert et al., 2005; Tokunaga and Esaka, 2007), no data are available on the molecular analysis of the 5′ promoter region of XIP-type inhibitor genes in these cereals.

Here we describe the molecular cloning and characterisation of a novel XIP-type inhibitor gene from wheat (*Triticum durum* Desf.). This is the first report of a complete gene sequence for a member of the XIP-type inhibitor family, providing insights into the regulation and physiological role of this class of inhibitors.

2. Experimental

2.1. Materials

The *Pichia pastoris* expression kit including the pHIL-D2 vector and GS115 strain was from Invitrogen (San Diego, CA, USA). Restriction endonucleases and DNA modifying enzymes were purchased from Promega (Madison, WI, USA) and used according to the manufacturer's recommendation. *Escherichia coli* DH5α (*supE*44, hsdR17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1) and XL1-Blue MRA(P2) strains (Stratagene) were used for DNA manipulation. Oligonucleotides were synthesized by Perkin–Elmer Applied Biosystems (Warrington, UK). S-200 chromatography, Hybond N+ filters and the Megaprime random priming kit were from GE Healthcare (Uppsala, Sweden). The GH11 xylanase C from *Penicillium funiculosum* (XYNC) was from in house (Furniss et al., 2002) and birchwood xylan from Fluka Chemicals (Poole, UK).

2.2. Isolation, sequencing and analysis of Xip-II genomic clone

A nucleotide probe was produced using nested PCR and degenerate primers (1st round primers F_n5 GBA ACA CBG GBC ARG TSA C and R_{II}5 ACV GTB GCV GTN AGR TG, 2nd round primers F_n3 GBC ARG TSA CBG TST TCT G and R_{ii}3 TGN AGS GGG TTV CCS GG) designed to XIP-I amino acid sequence (Elliott et al., 2002). This PCR product was used to screen a wheat (*T. durum* Desf cv Lira biotype 45) λDASHII genomic library. The lambda phage library was propagated in XL1-Blue MRA(P2) *E. coli* strain and plated out in NZY top agarose on NZY agar plates, with incubation at 37 °C. Duplicate plague lifts were performed on chilled plates containing 1.1×10^6 pfu, using Hybond N+ filters. Primary screens were performed using the Xip probe labelled with digoxigenin (DIG-dUTP nucleotide, Roche Applied Science) incorporated by PCR (D'Ovidio and Anderson, 1994); briefly, filters were removed from agar plates and soaked in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 1 min, then neutralising buffer (1.5 M NaCl, 0.5 M Tris–HCl pH 7.5) for 5 min, then dried and cross-linked (UV Stratalinker, Stratagene). Filters were re-hydrated in 2× SSC (0.3 M NaCl/0.03 M sodium citrate). Pre-hybridisation of the filters was performed in 4× SSC/1% (w/v) N-Lauroylsarcosine/0.02% (w/v) SDS/0.5% (w/v) blocking reagent (Roche Applied Science) for 3 h at 65 °C and then 16 h at 65 °C with DIG labelled probe. Hybridised filters were washed twice in 2× SSC/0.1% (w/v) SDS for 5 min at room temperature, then twice in 0.1× SSC/0.1% (w/v) SDS for 15 min at 65 °C, followed by single washes with 0.1 M maleic acid/0.15 M NaCl (pH 7.5) for 5 min at room temperature and 0.1× SSC/0.1% (w/v) SDS for 30 min at room temperature. Anti-DIG antibodies (Roche Applied Science) were then added to the wash solution and incubated at room temperature for 30 min, excess antibody was removed by washing twice with 2× SSC/0.1% (w/v) SDS for 15 min at room temperature. Filters were equilibrated in 0.1 M Tris (pH 9.5)/0.1 M NaCl/0.05 M $MgCl_2$ for 5 min at room temperature and bound antibody was detected using NBT (4-Nitro blue tetrazolium chloride) and X-Phosphate (Roche Applied Science).

Subsequent screens of positive plaques were performed using radiolabelled probes, labelled with (α-^{32}P-dATP) using the Megaprime random priming kit. Pre-hybridisation was performed in 6× SSC/0.5% (w/v) SDS/1× Denhardts reagent (0.02% w/v Ficoll/0.02% w/v polyvinylpyrrolidone/0.02% w/v bovine serum albumin containing 50 μg/ml sonicated herring sperm DNA as a blocking reagent), for 1 h at 60 °C. Probe hybridisation was then performed in 6× SSC/0.5% (w/v) SDS/1× Denhardts reagent/1 mM EDTA for 16 h at 60 °C. Positive plaques were identified by autoradiography.

Sequencing of positive plaques was performed directly from the phage DNA and was carried out with an Applied Biosystems model 373A automated sequencer using dye terminator chemistry by the DNA sequencing service (University of Durham).

Sequence analyses were performed using the DNAMAN software (Lynnon Biosoft, Quebec, Canada). Sequence alignments were performed using a gap open penalty of 10 and a gap extension penalty of 0.1. The tree was set up with the distance matrix using the UPGMA method.

The putative signal peptide and glycosylation sites were predicted using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP) and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc), respectively.

2.3. In silico *search of putative regulatory elements*

The TSSP-TCM program for the prediction of plant promoters and annotated promoters is available at http://mendel.cs.rhul.ac.uk/mendel. The *Xip*-II gene promoter sequences and 5′UTR were scanned for the presence of cis-elements identical with or similar to the motifs registered in two different plant cis-acting regulatory elements databases, PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE) and PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html), using the database associated search tools. Putative elements were checked for relevance, necessity of a repeat, distance between repeats, flanking sequences and distance from TATA-box, as outlined in the literature.

2.4. Functional expression of Xip-II in P. pastoris and purification of the recombinant protein

The cDNA encoding XIP-II was cloned into the *Eco*RI sites of the pHIL-D2 vector. Transformation of the *P. pastoris* strain GS115/(*his4*) and screening were achieved using the spheroplast procedure modified in Juge et al. (2001). Briefly, pHIL-D2/*Xip*-II (ca. 1 μg) as well as pHIL-D2 vector, as negative control, were digested with NotI prior to transformation. After screening for methanol sensitive clones, Mut^S colonies were used to inoculate 5 ml BMGY pH 6.0 and